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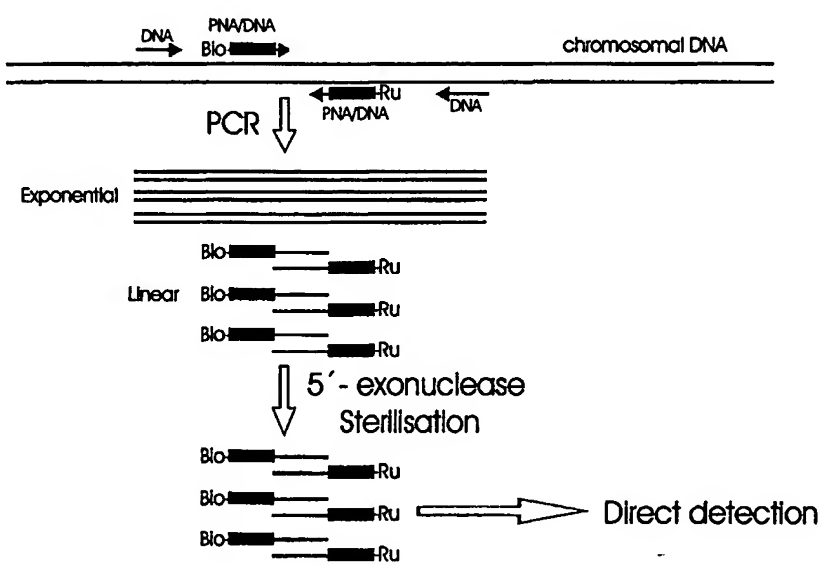

(57) Abstract

A method for the determination of nucleic acids is disclosed wherein in a first extension reaction a multiplicity of extension products of a first set of primers is produced, said extension products being digestible by nuclease, and in a second extension reaction extension products of a second set of primers are produced using said first extension products as templates, wherein said primers of said second set are not capable of serving as a template for the extension of a complementary primer. Intersample cross contamination can be reduced by subjecting said first extension products to nuclease treatment.

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A method for the determination of a nucleic acid

This invention is directed to a method for the determination of a nucleic acid using amplification of a partial sequence of said nucleic acid using two special sets of primers. a kit containing said sets of primers and a method for reducing intersample cross contamination.

Background of the invention

The determination of nucleic acids begins to play a more and more important role in analytical chemistry, especially in health care. Both infection diseases and genetic status can be determined relatively easily on the basis of the presence or the amount of a nucleic acid indicative of said disease or status in samples received from the individual. This was to a large extent made possible by the introduction of methods for the amplification of parts of the nucleic acids of interest. There are at least two different modes of primer based amplification, the first amplifying nucleic acid sequences in a cyclic manner theoretically exponential with the number of amplification cycles, based on the fact that each extension product of a primer or any product derived therefrom is used again as a template for the creation of a new extension product, which, in addition to the starting nucleic acid and the already formed extension product is subject to another round of extension using a second primer, and the other proceeding in a theoretically linear manner, such that each extension product is not used as a template in a further round, but only the original template. To the first group belongs the polymerase chain reaction (PCR), for example as described in US-A-4,683,202 and NASBA, as described in EP-A-0 329 822.

The PCR reaction allows minute amounts of target DNA to be amplified to detectable levels. To secure efficient amplification the target DNA is mixed with a large excess of two DNA primers and a large amount of polymerase enzyme and dNTP's. Whilst these conditions are mandatory to secure that efficient priming on the template occurs during successive cycles and that a successfully bound primer gets elongated it is also the

conditions that favours undesired side reactions. Hence, formation of artefacts such as primer dimers that occur as a result of transient formation of more or less matched primers and incorrect amplicons that occur as a result of primer binding to more or less matched sequences in the targed DNA constitute well documented problems in PCR.

When PCR is used as a diagnostic tool it is of course mandatory that the detection system is able to differentiate between the correct amplicons on the one hand and incorrect amplicons and primer dimers on the other hand. This can be achieved by using a detection system that generates either size information (the correct amplicon with high likelihood has a different size to that of incorrect amplicons and primer dimers) or even better, sequence information that allows an unambiguous assignment of the amplicon. An example of the latter case is disclosed in WO 89/10979. This format, although able to detect the correct amplicon with high certainty requires that the amplicons are denatured any hybridised to a detector probe prior to detection. Clearly, these steps prolongs the total assay time, complicates automation (both in terms of number of steps and the need for a heat controllable block that can assure specificity in the hybridisation between detector probe and amplicon) and increases the risk of inadvertently contaminating unrelated samples with amplicons.

Further there is known the so-called nested PCR, wherein two sets of primers are used, each of which contains two primer oligonucleotides the extension products of which can each serve as templates for the extension of another primer. The sets of primers are characterized in that the first set is designed to amplify a longer part of the nucleic acid and the second part being designed to amplify a subpart of said first part, thereby creating a multiplicity of shorter amplification products.

In WO 95/08556 there is disclosed a method of extending a chimeric molecule comprising a first nucleic acid analog part and an extendible second (nucleic acid) part using a nucleic acid to be determined as a template. This method does not create an exponential amount of extension products, because each extension product is not fully effective in serving as a template for the extension of a further primer. The reason for this is that after formation of the first extension product of the first chimeric primer, the second extension product formed based on using the first extension product as a template will get extended only to the end of the nucleic acid part of the first extension product, not forming a sequence complementary to the sequence of the nucleic acid analog part of the second primer. The small nucleic acid

part of the second extension product, being formed using the nucleic acid part of the first primer as a template, is too small to allow binding of a further molecule of said first primer to the second extension product stable enough for primer elongation. This method therefore is effectively linear.

In WO 96/40972 there is disclosed a method wherein a large number of nested linear amplifications are combined to yield an amplification rate, higher than in WO 95/08556. For each cycle a further set of primers is necessary. Thus the amplification power of the numer of possible method is limited by the nesting configurations. For instance, if the primary amplification product has a size of 300 bases only about 10 cycles of nested amplification can be performed using a primer length of 15 nt.

Recently, compounds were developed that can bind nucleic acids via nucleo base-pairing leading to the formation of stable complexes (WO 92/20702). Such compounds are generally acknowledged as not acting as templates for primer extension reactions. In the synthesis of the so called peptide nucleic acids (PNA), monomers are used containing a free or activated carboxylic acid group. They are reacted with an amino group on a solid phase which is either the starting functional group or is set free by deprotecting a monomer attached earlier in the synthesis. Thereby peptide bonds are formed between the monomeric subunits.

In EP-0 672 677 there is described a method for synthesis of nucleic acid analogues, wherein nucleotides and monomeric subunits containing a non-natural backbone are mixed. Those molecules are disclosed to be useful as probes for the determination of nucleic acids.

In J. Am. Chem. Soc. 1997, 119, 3177 - 3178, there is disclosed a method for the recognition of uncharged polyamide linked nucleic acid analogues by DNA polymerases and reverse transcriptass. It is further disclosed that linear amplification of the template strand was possible, but not exponential amplification of the target molecule. This was apparently due to the fact that the polymerases stopped evidently the enzymatic activity near the PNA-DNA linkage.

It is an object of the present invention to improve the methods for determination of nucleic acids, especially in avoiding all or a part of the disadvantages of the known methods.

It is another object of the invention to reduce the amount of primer-dimer extension products usually formed in cyclic amplification reactions.

It is another object of the present invention to provide a method for determination of nucleic acids which reduces the risk of intersample cross contamination.

Summary of the invention

A first subject is the combination of a first theoretically exponential amplification with a subsequent (second) theoretically linear amplification of a nucleic acid or parts thereof.

A further subject of the invention is a method for the determination of a nucleic acid in a sample comprising

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- producing a first set of extension products using a first set of primers being designed such that one of said primers is homologous to a partial sequence of said nucleic acid and another primer is complementary to another partial sequence of said nucleic acid located spaced apart from said first partial sequence such that the extension product of one primer is capable of serving as a template for the extension of the other primer, said nucleic acid serving as a template for the extension of at least one of said primers,

- producing a second set of extension products using extension of a second set of primers designed such that a first of said primers is homologous to one strand of said first extension products within a region produced by said first extension reaction of one of the primers of said first set and a second primer is complementary to another region of said strand produced by said first extension reaction, and

- determining the presence or amount of said second extension products as a measure for the presence or amount of the nucleic acid in said sample,

- wherein the first primer of said second set is essentially not capable of serving as templates for the extension of the second primer of said second set.

Brief description of the drawings

FIG 1 shows schematically a preferred process according to the invention.

Detailled description of the invention

A primer according to the present invention is a molecule capable of being extended enzymatically, preferably by a polymerase of for instance procaryotic origin, when hybridized to a nucleic acid template. Preferred are thermostable polymerases, like T. aquaticus DNA- polymerase. The extension adds mononucleotide units from monodesoxyribonucleosidetriphosphates to one end of said primer, preferably the 3'-OH-terminal end. The overall length and base sequence of a primer is dictated by the required specificity of the amplification reaction. Preferred primer lengths are from 12 to 30, most preferred from 15 to 25 base containing subunits, selected from mononucleotides and/or nucleic acid analog monomers. Preferably the primers of each set cannot hybridize to each other, because they do not contain any stretch of more than 5 consecutive complementary bases, both within each set and between the different sets.

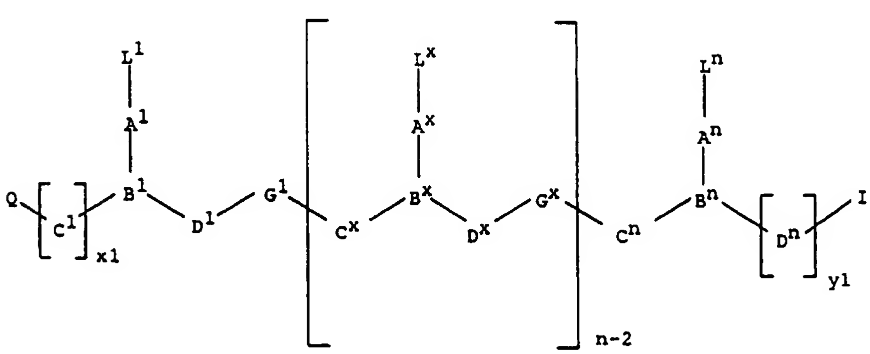
The locations of hybridisation of the primers of the first set are choosen such that there is a stretch of at lest 50, but not much more than 500, preferably from 100 to 300 nucleotides between their original 3'-ends, in the hybrid of the extension products. Practically the primers of the first set are oligonucleotides.

The stretch for the second set is smaller, starting with 0, preferably 3 to 300, most preferably 5 to 100. The primers of said second set are no pure oligonucleotides, but contain a stretch of at least 10, preferably more than 12, and no more than 30, base containing units starting with a moiety interrupting, preferably stopping, the ongoing extension of the extension product of a primer, preferably a non-natural base containing unit from a nucleic acid analog monomer. They contain at one end at least one free enzymatically extendible hydroxyl group, more preferably a stretch of from 1 to 8, most preferably from 1 to 3 base containing units that can be enzymatically extended and in principal be template for primer extension reactions, preferably mononucleotide units with a free 3'-hydroxyl group at the end. The non-natural base containing unit is not a template under the conditions of the amplification performed with the first and second set of primers

Nucleic acid analog monomers are the non-naturally occurring monomers of nucleic acid binding compounds which are molecules capable of binding to nucleic acids via base-pairing. Such base-pairing usually occurs by hydrogen bonding between complementary

nucleobases, like A base pairing with T or U as well as C base pairing with G. Such nucleic acid binding compounds may be known-in-the-art, preferred are those from WO 92/20702. Not only PNA with identically repeating backbone moieties are encompassed by the present invention, but also nucleic acid binding compounds, wherein the backbone part is made up by different monomeric backbone subunits, for example aminoethylglycine and aminoethyllysine. Such compounds are described, for example, in WO 95/14706, the content of which is hereby incorporated for defining the nucleic acid binding compound structure. Further encompassed are compounds as disclosed in EP-A-0 672 677, wherein there are disclosed mixed structures of peptide bonded monomers and oligonucleotide subunits. Further encompassed as nucleic acid binding compounds are compounds as disclosed in WO 96/20212 and EP-A-0 700 928.

The preferred primers of the second set are preferably characterized in that they comprise ligands linked to a pseudo peptide backbone via an aza or tertiary amino nitrogen atom within said backbone. It is most preferred that besides the aza nitrogen atom, at least some of the monomeric subunits are connected to each other using amide bonds (-CONH-). The backbone of the nucleic acid binding compound is preferably a moiety containing subunits arranged in a linear manner, to which subunits the ligands may be bound at different sites preferably at locations being separated from each other by between 4 and 8 atoms. Preferred primers are shown in formula I:



Formula I

wherein

n is an integer of from at least 3,

x is an integer of from 2 to n-1,

each of L^1-L^n is a ligand independently selected from the group consisting of hydrogen, hydroxy, (C_1-C_4)alkanoyl, naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, reporter ligands and chelating moieties, at least one of L^1-L^n containing a primary or secondary amino group;

each of C^1-C^n is $(CR^6R^7)y$ (preferably CR^6R^7, CHR^6CHR^7 or $CR^6R^7CH_2$) where R^6 is hydrogen and R^7 is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R^6 and R^7 are independently selected from the group consisting of hydrogen, (C_1-C_6)alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C_1-C_6)alkoxy, (C_1-C_6)alkylthio, NR^3R^4 and SR^5, where R^3 and R^4 are as defined below, and R^5 is hydrogen, (C_1-C_6)alkyl, hydroxy, (C_1-C_6)alkoxy, or (C_1-C_6)alkylthio-substituted (C_1-C_6)alkyl or R^6 and R^7 taken together complete an alicyclic or heterocyclic system; or C^1-C^n is CO, CS, CNR^3;

each of D^1-D^n is $(CR^6R^7)_z$ (preferably CR^6R^7, CHR^6CHR^7 or $CH_2CR^6R^7$) where R^6 and R^7 are as defined above;

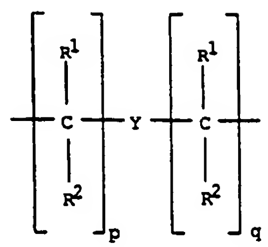
each of y and z is zero or an integer from 1 to 10, the sum y + z being at least 2, preferably greater than 2, but not more than 10;

each of G^1-G^{n-1} is $-NR^3CO-$, $-NR^3CS-$, $-NR^3SO-$ or $-NR^3SO_2-$, in either orientation, where R^3 is as defined below;

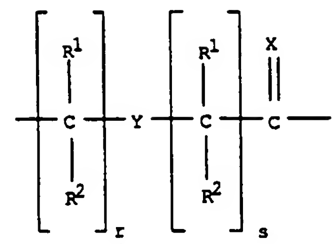
each of A^1-A^n and B^1-B^n are selected such that:

(a) A^1-A^n is a group of formula (I/A), (I/B), (I/C) or (I/D), and B^1-B^n is N or R^3N^+; or

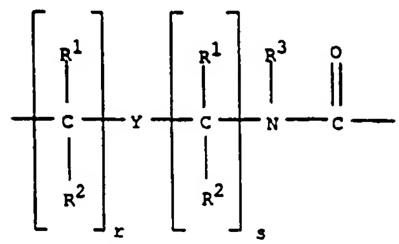
(b) A^1-A^n is a group of formula (I/D) and B^1-B^n is CH;



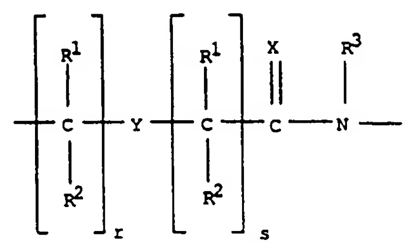
Formula I/A



Formula I/B



Formula I/C



Formula I/D

wherein:

X is O, S, Se, NR^3, CH_2 or $C(CH_3)_2$;

Y is a single bond, O, S or NR^4;

each of p and q is zero or an integer from 1 to 5, (the sum p+q being preferably not more than 5);

each of r and s is zero or an integer from 1 to 5, (the sum r+s being preferably not more than 5);

each R^1 and R^2 is independently selected from the group consisting of hydrogen, (C_1-C_4)alkyl which may be hydroxy- or (C_1-C_4)alkoxy- or (C_1-C_4)alkylthio-substituted, hydroxy, (C_1-C_4)alkoxy, (C_1-C_4)alkylthio, amino and halogen; and

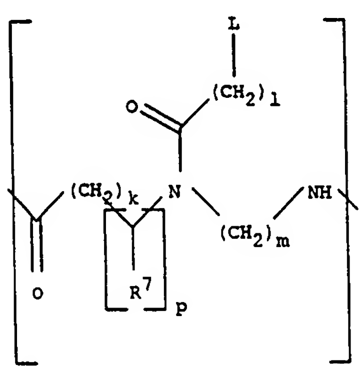
each R^3 and R^4 is independently selected from the group consisting of hydrogen, (C_1-C_4)alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C_1-C_4)alkyl, hydroxy, (C_1-C_6)-alkoxy, (C_1-C_6)-alkylthio and amino;

one of Q and I being selected from the group consisting of NH_2, $CONH_2$, COOH, hydrogen, (C_1-C_6)-alkyl, O-(C_1-C_6)-alkyl, amino protected by an amino protecting group, carboxy protected by a carboxyl protection group, reporter ligands, intercalators, chelators, peptides, proteins, carbohydrates, lipids, steroids, nucleosides, nucleotides, nucleotide diphosphates, nucleotide triphosphates, oligonucleotides, including both oligoribonucleotides and oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers as well as nucleic acid binding moieties,

the other of Q and I being defined as selected from the group of a hydroxyl group and a moiety having a free enzymatically extendible terminus, preferably a hydroxyl group, like from a mononucleotide or an oligonucleotide and

each of x1 and y1 is an integer of from 0 to 10.

Most preferred primers of the second set comprise at least one monomeric subunit of the general formula II:



(II)

wherein

L is a ligand as defined above for L^1 -L^n,

k, l and m is independently zero or an integer from 1 to 5,

p is zero or 1, and

R^7 is selected from the group consisting of hydrogen, C_1-C_3-alkyl and the side chains of naturally occurring alpha amino acids.

One or more of the primers of said second set can be coupled to labels, preferably at a location not substantially interfering with the primer's ability to get extended. Preferred binding sites of labels are either at the backbone or the bases of the analog part of the primer. Most preferred is the final end group, for example an amino group of a PNA.

Ligands are preferentially selected from the group consisting of hydrogen, phenyl, fused aromatic moities, heterocyclic moities, naturally occurring nucleobases and non-naturally occurring nucleobases, either unsubstituted or substituted by a reporter group, as well as reporter groups. Naturally occurring nucleobases are for example the main nucleobases like thymine, cytosine, adenine and guanine or rare bases like inosine, 5-methylcytosine or thiouracil. An example of a non-naturally occurring nucleobase is 7-deaza-guanine. An example of a fused aromatic moiety is naphthol. A heterocyclic moiety is pyridin. Reporter groups are moieties that can be detected (labels), like fluorescent compounds, for example fluorescein, or moieties that can be recognized by another molecular entity, like haptens which can be recognized by an antibody raised against this hapten or moieties that can be immobilized, like biotin (to streptavidin coated solid phases, like tubes or beads). In order to achieve binding of the nucleic acid binding compound to the nucleic acid, the nucleic acid binding compound generally will comprise a sufficient number of nucleobases as ligands for base pairing to nucleobases of the nucleic acid, for example at least 6 nucleobases. At the end of the backbone having the nucleobases attached, the backbone may contain ligands other than nucleobases.

A label or reporter group is generally known to a man skilled in the art as being a group which is detectable or can be made detectable for determining the presence of the nucleic acid binding compound. Well-known labels are fluorescent labels, like fluoresceine, electrochemoluminescent labels, like ruthenium complexes, biotin or digoxigenin. Labels can either be used for detection of the primer or any product having incorporated said primer.

The nucleic acid to be determined can be the analyte nucleic acid. This can be of any origin, for example of viroid, viral, bacterial, or cellular origin. It can be derived from solutions,

like blood, serum, plasma or urine, from suspensions, fixed on solids, cell containing media, cell smears, fixed cells, tissue sections or fixed organisms. Preferably the nucleic acid to be determined is in solution. Nucleic acid to be determined can further be a nucleic acid derived from the analyte nucleic acids , for example by recombination, fragmentation or cDNA formation from RNA. The nucleic acid to be determined usually has a length of at least 40 nt.

The analyte nucleic acids usually are brought into available form from their containments by treatment with various reagents. This comprises for example change of pH (alkaline), heating, cyclic changes of temperature (freezing/thawing), change of the physiological growing conditions, use of detergents, chaotropic salts or enzymes (for example Proteases or lipases), alone or in combination.

A template is generally defined as a nucleic acid which can direct the synthesis of a complementary nucleic acid strand, for example by a DNA polymerase or an RNA polymerase. The DNA polymerase will add mononucleotides to a primer hybridized to the template whereby the primer is extended by bases complementary to the corresponding bases on the template.

A nucleic acid or nucleic acid analog essentially complementary to another nucleic acid or nucleic acid analog is defined to contain a sequence of bases enabeling it to bind to the other nucleic acid or analog by base pairing. Total complementarity is given, if the nucleic acid or analog contains a stretch of at least 10 consecutive bases base pairing with a stretch of at least 10 consecutive bases of the other nucleic acid or analog.

A probe is a molecule used for the determination of extension products. These probes are therefore complementary to a part of the extension product to be determined, especially in the part of the extension product not contained in the primer, but newly formed by adding mononucleotides to the primer. The probe can be advantageously an oligonucleotide, but also an analog, for example a PNA.

A solid phase is a solid not substantially soluble in the reaction mixture, for example in the form of a bead, a net, the inner surface of a tube, a microtiter plate or a chamber of a device. It is essentially used to contain the reaction mixture, but in case of intention to bind

an immobilisable probe or primer to it, it may contain at its surface reagents or a coating being capable to recognize and bind a moiety of said probe or primer.

Preferably, theoretically exponential amplification is achieved by the PCR reactions. The primers of the first set of primers are therefore designed such that each extension product formed from said primers can act over their full length, especially in the primer originating parts, most preferably over a length of at least 10 nucleotides (nt) calculated from the originally 3'-end of said primers, as templates for the extension of the other primers of the set. Theoretically exponential amplification can also be achieved via transcription based amplification, the amplification rate being enhanced by a factor based on the promotor controlled transcription of DNA into RNA. Such a method is the so called NASBA.

A theoretically linear amplification is preferably a cyclic amplification, in which the products of an earlier amplification cycle are not used for further amplification.

In the first part of the method according to the present invention there is produced in a theoretically exponential manner a first set of extension products using the first set of primers. This part is proceeding essentially as known from the art, for example from PCR. There is no essential difference in reagents or reaction conditions from known methods. In a second part, however, a theoretically linear amplification is performed using extension products formed in the first part as starting material for the extension of the second set of primers.

In the first cycle of the second extension reaction using any of the extension products of the first set as a template for the extension of one of the primers of the second set there are produced extension products being complementary to the other primer of said second set within the region formed from mononucleotides, such that the other primer can hybridize to this extension product. This is made for both primers of said second set. In the next cycle of extension, after separating the extension products from their templates the extention products of said primers of the second set are available for hybridization with the respective other primer of said second set. Extension of these other primers leads to a second set of extension products their length being defined such as to include the primer and a stretch of newly added mononucleotides, stopping essentially with the mononucleotide complementary to the last base at the position contiguous to the first non-natural monomer

subunit or the moiety stopping polymerase activity. The remaining part of the primer is not used as a template for primer extension.

It is not excluded that, especially when further primers are used in addition to the primers of the first and second set, further products are produced. However, it is a preferred embodiment of the present invention that only the extension products of the second set of primers produced by using only extension products of said first or second set of primers as templates are determined as a measure of the nucleic acid to be determined. This can be achieved by the present invention because of the particular choice of molecular structure of the second set of primers, which reduces or even prevents the formation of extension products of primers of the second set using primers as templates (based on the formation of primer dimers). Even if by chance a partial primer dimer is produced by hybridizing a first primer of said second set to another primer of said second set, it is highly likely that it will not be extended, as a substantial part of the other primer is not acting as a template, or it will be extended only by a very limited amount of mononucleotides which is too small in order to maintain hybridization of the primer dimer formed under the conditions used.

For the above it follows that the first and second amplification can be performed in a subsequent manner, for example performing the exponential first part as long as required as to substantially increase the amount of sequences to be basis for the determination, for example for 5 to 50, preferably 10 to 30 cycles, and thereafter add the reagents (especially the primers of the second set) for the linear amplification, and perform the linear amplification for as many cycles as required to produce a readily detectable amount of hybrids of extension products of the second set. The number of cycles required is generally lower than in the exponential reaction. Preferably the number of cycles is from 2 to 20, most preferably from 3 to 10, but a higher number usually do not affect the success of the determination.

If the conditions of the second part of the method are also favourable for the exponential amplifications the sensitivity of the assay will of course further increase.

In another embodiment the reagents for the second part can also be added after very few (1 to 5) cycles of the exponential amplification or even be added at the start or together with the reagents for the exponential amplification. Then it will be generally preferred to perform

some cycles in addition to the number of cycles that are usually performed in exponential amplification. However, this may depend upon the sensitivity requested in the assay. This simultaneous approach is preferred to the sequential approach, because it avoids contaminating the environment with amplificates when opening the reaction tube for adding reagents.

The hybrids can be determined by methods generally known in the art, for example hybridization with a detectably and/or immobilizably labelled probe, for example according to EP-B-0 079 139 or US-5,476,769. However, in preferred embodiments at least one of the primers of the second set is labelled. The label can be used to determine the hybrid. In this case it may be advantageous to separate any extension products having label incorporated via the primer from any excess of labelled primer. this can either be made via immobilizing the labelled extension product to a solid phase by sequence specific capture using a probe hybridizing in the extended region outside the primer hybridisation positions and removing the remaining liquid containing the primers. In a preferred mode of the invention, however, one of the primers (forward) of the second set is detectably labelled and the other (reverse) is immobilized to a solid phase or immobilizibly labelled. Such that the extension products having the reverse primer incorporated can be used to bind any extension products incorporating a detectable label to a solid phase. The excess of (unincorporated) labelled primers is removed then prior to determination.

The determination can be made by measuring the amount of label present on the solid phase according to known methods, for example by applying ELISA techniques to recognize the label, or by direct fluorimetry. The embodiment using both detectable and immobilizable primer has the advantage that no additional capture probe, no denaturation step and no hybridization step is necessary.

In a further preferred embodiment the reaction mixture after the amplification reaction is subjected to a step that digests the products of the exponential amplification, while not substantially destroying the products of the linear amplification. This differentiation can be made by using primers of the second set which are protected against the digestion such that the extension products of the second part are still detectable. This can be achieved by using non-natural monomer subunits. There are known now some modified subunits, for example

subunits being connected via peptide bonds, that are resistant to all nucleoses. Preferred digestion is via an exonuclease (for example attacking nucleic acids from the 5'-end).

This will effectively destroy all amplicons generated from the first set of primers thereby substantially eliminating the risk of intersample cross contamination, that could lead to substantial false positive results in samples being performed in the neighbourhood or even in the same room.

A preferred embodiment of the invention is described in FIG. 1. Therein the first set of primers is composed of oligonucleotides, while the second set is composed of two chimeric primers having a ruthenium or biotin label at their non-extendible ends, respectively. The mixture is subjected to PCR conditions, whereby a large amount (theoretically exponential) of extension products of the first primers is formed. As the second set of primers can use the first extension products as templates, for each extension product present in the mixture one extension product of one primer of the second set is formed. These extension products will not serve as templates for the extension of the other primer of said second set, because the oligonucleotide part of said other primer will not sufficiently support hybridisation to the extension product of the other primer. The second amplification, based on said second set is therefore linear, and the second extension products are merely accumulated in the reaction mixture. Nuclease digestion destroys substantially all extension products of the first set. The extension products of the second set are in the embodiment then contacted with a solid phase being capable of binding said immobilisable group I. The presence or amount of extension product formed is then detected via the label D in the hybrid bound to the solid phase. This can be taken as an indication or measure for the presence of the analyte nucleic acid in the sample.

A further subject of the present invention is the use of primers being protected against 5'-nuclease attack for avoiding intersample nucleic acid cross contamination in amplification reactions.

A further subject of the present invention is a method for avoiding an intersample nucleic acid cross contamination comprising producing first amplification products from a partial sequence of nucleic acids in a sample, forming second amplification products being

protected against 5'-nuclease attack using said first nucleic acid amplification products as templates, digesting said first nucleic acid amplification products with a 5'-nuclease.

A further subject of the present invention is the use of primers being essentially not capable of serving as templates for the extension of primers of different complementarity for avoiding the formation of amplification products from primer-dimers.

The present invention is exemplified by the following examples:

Examples

General

All reagents used are analytical grade and from Aldrich. HATU was purchased from PerSeptive Biosystems (MA). MBHA resin were purchased from NovaBiochem (Switzerland). EZ-Link™NHS-SS-Biotin was from Pierce (IL). Bis-(2,2'-bipy)-4-(4-(4'-Me-2,2'-bipy))-butane Acid-Ruthenium (II)-bis-hexafluoro Phosphate was purchased from Boehringer Mannheim GmbH. N-MMT-(CH_2)-aminoModifier was from Clontech Palo Alto(CA,USA). The oligomers used in the examples are linear and single stranded.

Abbreviations:

HOBt:	1-Hydroxybenzotriazole
DCM:	Di-chloromethane
MBHA:	Methyl-benzhydrylamine
PAM:	Phenylacetamidomethyl
HATU:	O-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophasphate
DIEA:	Diisopropylamine
Ado:	8-Amino-3,6,-dioxa-octanoic acid

Example 1

Synthesis of oligomers having a PNA and an oligonucleotide part (chimeric molecules; chimera)

The DNA part was synthesized using standard phosphoramidite synthesis on a CPG support. The introduction of the 5'-amino functionality, was achieved using a special 6-(Mmt-amino)hexyl phosphoramidite. The support was transferred to a reaction vessel, and the Mmt group was removed during treatment with 3 % trichloroacetic acid in DCM. The PNA monomers were added using the above described cycle. The synthesis was also achieved on a ABI 433A peptide synthesizer using methods in which the vortex has been exchanged with nitrogen purging, to be able to use DNA on CPG resin.

Ruthenium complexes were coupled to the N-terminus of the distal PNA monomer by activation of the carboxylic group on the Ruthenium conjugand by HATU, biotin was also activated by HATU but double coupled, fluoresceinisothiocyanate (Fitch) was coupled for 24 h without further activation and with only catalytic amount of DIEA. When biotin or fluorescein was coupled, the final PNA monomer was not capped with acetic anhydride.

The final chimera was removed from the support and deprotected using either ammonia in water (32 %) for 20 h at 60 °C or with methylamine (40 %) at room temperature for 4 h.

Example 2

Use of the primers as polymerase substrates

The template MKA1 (5'-AGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGG-3', SEQ.ID.NO. 1) was hybridised to either an equimolar amount of the DNA primer MKA5 (5'-CCTGATAGACGGTTTTT-3', SEQ.ID.NO. 2) or the PNA-DNA chimera, PNA 633-bio (Bio-CCTGATAGACGG-NH-$(CH_2)_6$-OPO_2HO-**TTTTT-3'**, the DNA segment is shown in **bold**).

Template MKA2 (5'- CTTTGACGTTGGAGTCCACGTTCTAAAAAATGAGCTGATTTAACA-3', SEQ.ID.NO. 3) was hybridised to either an equimolar amount of the DNA primer MKA4 (5'-TAAATCAGCTCATTTTT-3', SEQ.ID.NO. 4) or the following PNA-DNA chimeras, PNA 634-Fitch (Fitch-TTAATCAGCTCA--**TTTTT-3'**), PNA 635-Bio (Bio-TGTTAAATCAGCTCA--**TTTTT-3'**), PNA 636-Ru (Ru-TGTTAAATCAGCTCA-NH-$(CH_2)_6$-OPO_2HO-**TTTTT-3'**). Primer extension reactions (50 µl) were performed in the presence of ^{32}P dATP using either the Klenow exopolymerase (buffer supplied by the manufactor of the enzyme) or the B.st. polymerase buffer supplied by the manufactor of the enzyme). Reactions containing the Klenow-exo-enzyme were incubated at 30 °C for 30 min. whereas reaction containing the Bst enzyme were incubated at 50 °C for 30 min. The reactions were stopped by the addition of formamide containing loading buffer and 10 ml of the stopped reactions were loaded onto 6 % denaturating polyacrylamide gels. After

electrophoresis the gels were subjected to autoradiography for 1 hour. The results are given in FIG. 2

As evident from the figure all PNAs functions as primers with the selected enzymes (lane 1 to 8). When the corresponding DNA oligos are used instead of the PNA-DNA chimeras primer extension products are also obtained (lane 9 to 12). Since these products are interily composed of charged DNA subunits one would expect their mobility to be slightly bigger than the corresponding products obtained with the PNA-DNA chimeras (the PNA extension products contains 12 charges less than the DNA primed products). Indeed, this expectation is corroborated by the experiment which clearly shows that the DNA primed products migrates faster in the gel than the PNA primed products (compare lane 1 to lane 9; lange 2 to lane 10, lane 3 to lane 11 and lane 4 to lane 12). When no primer (PNA or DNA) is added to the template no visible primer extension product is obtained in the case of the MKA1 template (lanes 13 and 14). Some weak bands are obtained with the MKA2 template indicating that some self-priming is possible with this template. These bands, however, has a different mobility from the bands produced with either the PNA or the DNA primers.

We conclude that all of the four PNA-DNA chimeras works efficiently as primers for the template dependent DNA polymerases, Klenow exo- and Bst polymerase (large fragment). Moreover, we conclude that the attachment of different labels (Ru and FITC) or ligands (biotin) to the PNA part of the chimera does not prevent its function as a primer.

Claims

1. A method for the determination of a nucleic acid in a sample comprising

   - producing a first set of extension products using a first set of primers being designed such that one of said primers is homologous to a partial sequence of said nucleic acid and another primer is complementary to another partial sequence of said nucleic acid located spaced apart from said first partial sequence such that the extension product of one primer is capable of serving as a template for the extension of the other primer, said nucleic acid serving as a template for the extension of at least one of said primers,

   - producing a second set of extension products using extension of a second set of primers designed such that a first of said primers is homologous to one strand of said first extension products within a region produced by said first extension reaction of one of the primers of said first set and a second primer is complementary to another region of said strand produced by said first extension reaction, and

   - determining the presence or amount of said second extension products as a measure for the presence or amount of the nucleic acid in said sample,

   - wherein the first primer of said second set is essentially not capable of serving as templates for the extension of the second primer of said second set.

2. A method according to claim 1, wherein one of said primers of said second set is labelled immobilizably.

3. A method according to claim 2, wherein the second primer of said second set is labelled detectably.

4. A method according to claim 1, wherein said primers of said second set are protected against 5'-nuclease attack.

5. A method according to claim 2, further comprising contacting the second extension products with a solid surface capable of binding said immobilizable label.

6. A method of claim 1, wherein the second set of primers contains a stretch of nucleic acid analog monomer subunits.

7. Method of claim 6, wherein said nucleic acid analog is a peptide nucleic acid.

8. A method of any of claims 1 to 7, wherein the extendable ends of said primers of said second set contains of from 1 to 3 mononucleotide subunits.

9. A method of any of claims 1 to 8, further comprising subjecting said first extension products to a 5'-nuclease treatment prior to the determination of said second extension products.

10. The use of primers being protected against 5'-nuclease attack for avoiding intersample nucleic acid cross contamination in amplification reactions.

11. A method for avoiding an intersample nucleic acid cross contamination comprising

- producing first amplification products from a partial sequence of nucleic acids in a sample,
- forming second amplification products being protected against 5'-nuclease attack using said first nucleic acid amplification products as templates,
- digesting said first nucleic acid amplification products with a 5'-nuclease.

12. The use of primers being essentially not capable of serving as templates for the extension of primers of different complementarity for avoiding the formation of amplification products from primer-dimers.

FIG 1

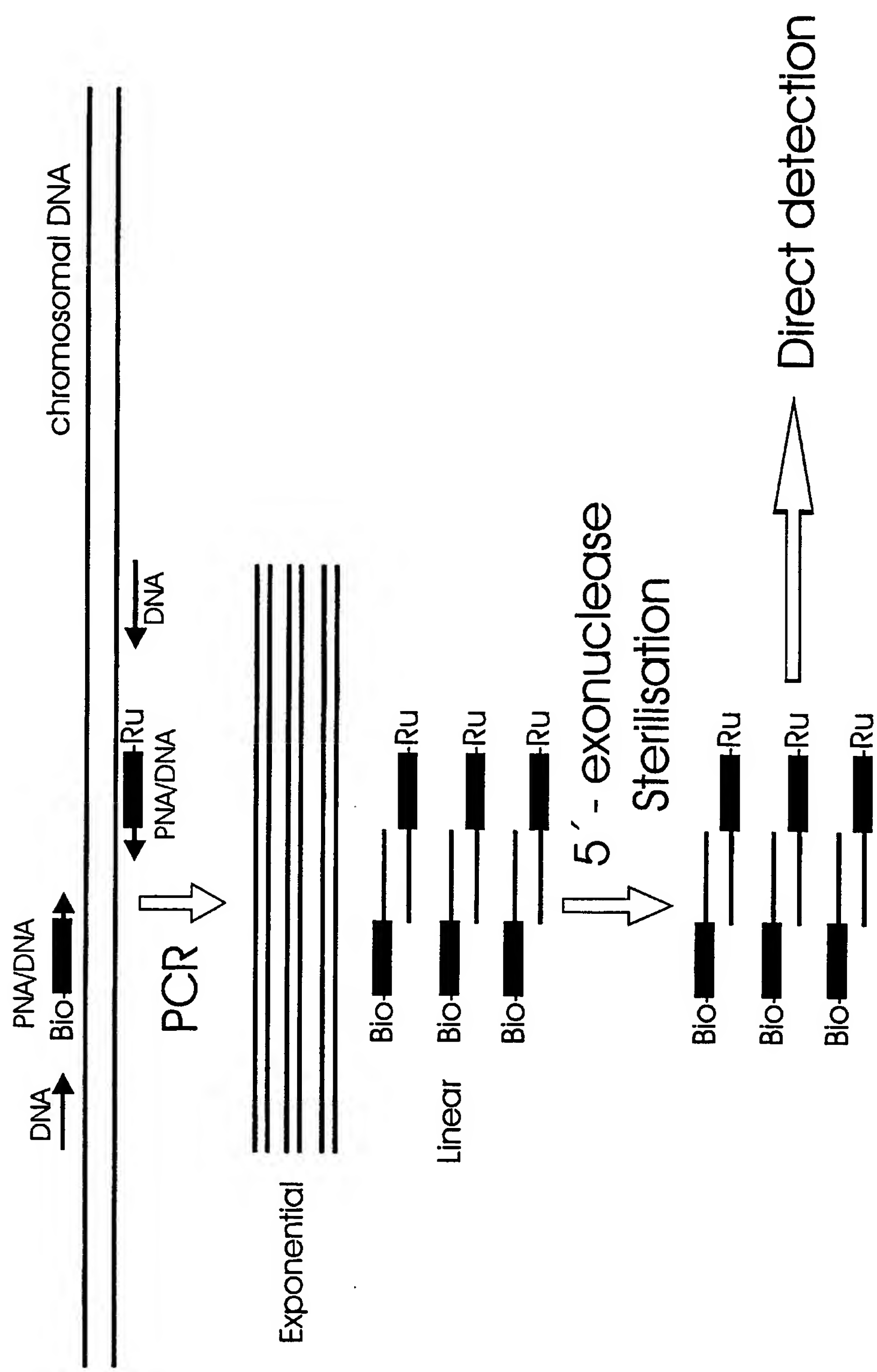
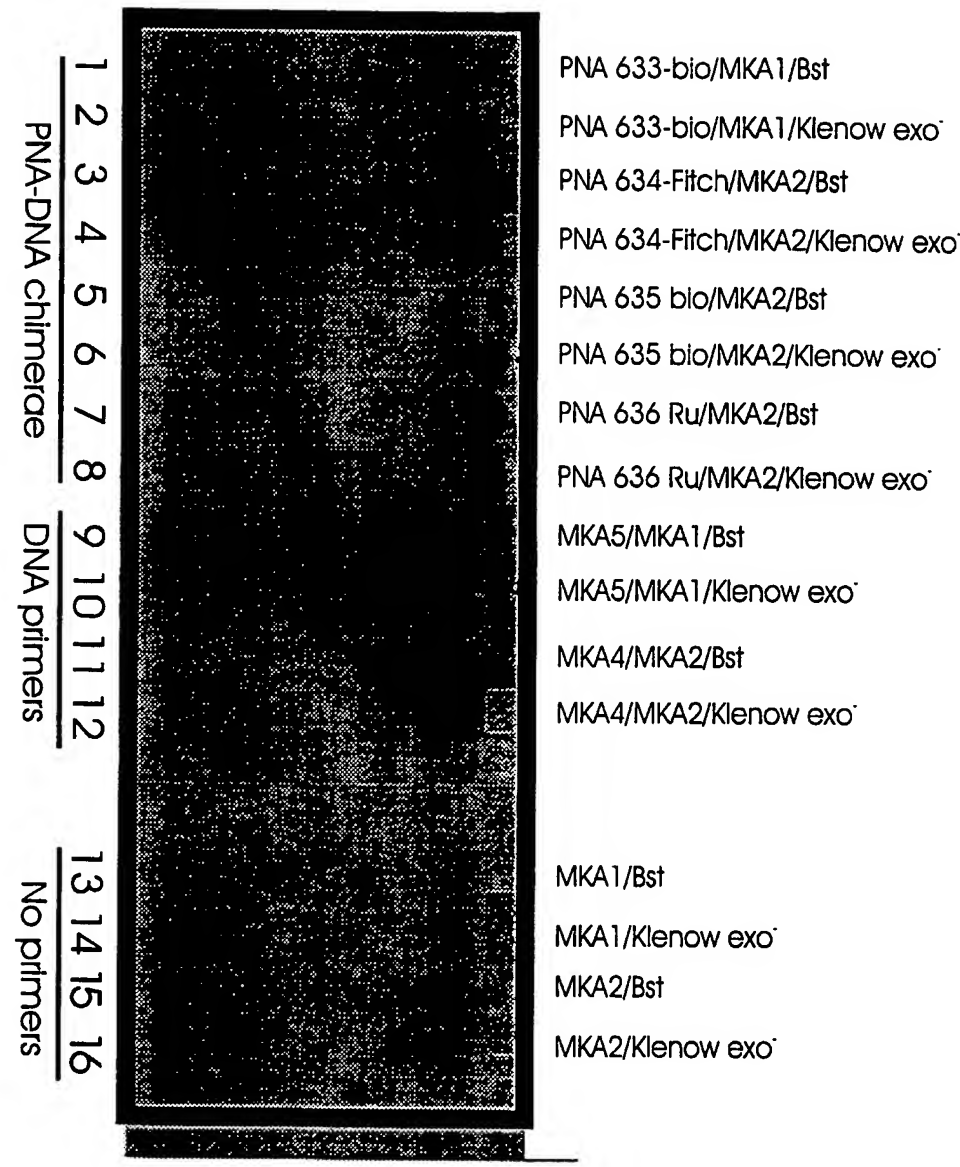

chromosomal DNA
DNA
PNA/DNA
Bio
Ru
DNA
PNA/DNA
Ru
PCR
Exponential
Bio
Bio
Bio
Ru
Ru
Ru
Linear
5´ - exonuclease
Sterilisation
Bio
Bio
Bio
Ru
Ru
Ru
Direct detection

FIG 2


PNA-DNA chimerae
DNA primers
No primers
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PNA 633-bio/MKA1/Bst
PNA 633-bio/MKA1/Klenow exo⁻
PNA 634-Fitch/MKA2/Bst
PNA 634-Fitch/MKA2/Klenow exo⁻
PNA 635 bio/MKA2/Bst
PNA 635 bio/MKA2/Klenow exo⁻
PNA 636 Ru/MKA2/Bst
PNA 636 Ru/MKA2/Klenow exo⁻
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MKA1/Bst
MKA1/Klenow exo⁻
MKA2/Bst
MKA2/Klenow exo⁻

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Boehringer Mannheim GmbH

(B) STREET: Sandhoferstr. 116

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(E) COUNTRY: DE

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(ii) TITLE OF INVENTION: A method for the determination of a nucleic acid

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligodesoxyribonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGAACGTGGA CTCCAACGTC AAAGGGCGAA AAACCGTCTA TCAGG 45

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligodesoxyribonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCTGATAGAC GGTTTTT 17

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Desoxyribonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTTTGACGTT GGAGTCCACG TTCTAAAAAA TGAGCTGATT TAACA 45

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Desoxyribonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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